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Colorimetric Determination of Chloride in Biological Samples by Using Mercuric Nitrate and Diphenylcarbazone

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ABSTRACT

A colorimetic method is outlined for the determination of the chloride ion in biological samples (blood serum, plasma, and urine). The present method is based on the quantitative reduction of free mercuric ions by chloride ions. Chloride ions form an indissociable complex with mercuric ions. The remaining free mercuric ions form a purple complex with diphenylcarbazone with an absorption maximum at 550 nm. The reduction of color intensity at 550 nm is directly proportional to chloride concentration in the sample. The linear concentration range in the final reaction mixture was 0–100 μ*M* with a correlation coefficient of –0.9997. The coefficient of variation for the 50 μ*M* chloride ion in the final reaction mixture was 0.9% (n = 6). The analyzed value of chloride concentration in the human control serum Accutrol™ Normal (Sigma) was 101±4 m*M* (mean±SD, n = 12). The certified value of chloride in Accutrol Normal by Sigma is 102 m*M,* with a mean in the range 91–113 m*M.* This method was applied to the

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measurement of urinary chloride excretion in experimental rats. During 16-h urine collection, no food was given and rats had free access to purified water. The urinary excretion rate of chloride was 23.6±9.3 μmol/h (mean±SD, n = 8) and 126.2 ± 28.0 μmol/h (n = 8) for rats fed a normal diet (2.6 g NaCl/kg diet) and a high-salt diet (82.6 g NaCl/kg diet) for 70 d prior to urine collection, respectively. This method is appropriate for low concentrations of chloride in samples or when sample volume is limiting, as in many animal studies such as metabolic urine collection from rats.

Index Entries: Chloride ion; determination; mercuric nitrate; diphenylcarbazone; colorimetry; plasma; serum; urine; human; rat.

INTRODUCTION

Plasma or serum chloride is routinely assayed by the colorimetric method of Schoenfeld and Lewellen *(1)*, which utilizes iron–thiocyanate complex formation, by a clinical chloride ion meter that uses a chloride ion-selective electrode *(2)*, or by cyclic voltammetry *(3)*. In the colorimetric method of Schoenfeld and Lewellen *(1)*, the chloride ions in samples combine with mercuric ions to liberate thiocyanate ions in mercuric thiocyanate and the liberated thiocyanate ions combine with ferrous ions to produce the color. Because of the relatively lower sensitivity and nonlinearity of the colorimetric methods using the iron–thiocyanate complex *(1,4)* and the difficulty in tuning of the chloride meter that is usually specialized for human serum or plasma in clinical laboratories, determination of urinary chloride is based on titration methods using the reaction between chloride and mercuric ions *(5)* or between chloride and silver ions *(6)*. In the mercuric nitrate titration method of Schales and Schales *(5)*, the chloride ions in samples combine with the mercuric ions of the mercuric nitrate to form soluble but virtually indissociated mercuric chloride. After all of the chloride ions are combined, the next drop of mercuric nitrate added will release free mercuric ions, which will cause the color change of the indicator diphenylcarbazone. In the silver nitrate titration method *(6)*, potassium dichromate is used as an indicator in place of diphenylcarbazone. There is difficulty in judging the correct end point, especially for the silver nitrate method. The coulometric titration method solved this problem but requires the special instrumentation *(7)*.

In animal studies, the volume of urine is low and the urinary chloride concentrations are lower compared to urine of humans who usually consume an appreciable amount of salt in their diets. Excessive dietary sodium chloride is an important risk factor of essential hypertension and the reduction of dietary sodium chloride is one of the most effective treatments of essential hypertension *(8)*. Animal studies were performed to investigate the mechanisms of salt (sodium chloride)-induced hypertension *(9,10)*. Differential effects of sodium and chloride ions on blood pressure control are still not well established because investigations were usually limited to the determination of sodium ion *(11,12)*. Thus, there is a

need to develop a sensitive method for the determination of chloride that is applicable to animal urine specimens.

A simple colorimetric method for the determination of chloride ions in biological samples is reported. The method uses the inhibition of color complex formation between the mercuric ion and diphenylcarbazone through the indissociable complex formation between the mercuric ion and the chloride ion. This method is appropriate for low concentrations of chloride in samples (above 0.4 m*M*) or when the available sample volume is low, as in many animal studies.

MATERIAL AND METHODS

Principle

In the present method, the chloride anion is measured by reciprocal colorimetry. Reagent A contains mercuric cation (Hg^{2+}) that binds chloride anion (Cl–) in the sample solution to form indissociable $HgCl_2$. When the sample solution is mixed with Reagent A, the amount of dissociated Hg^{2+} is decreased depending on the amount of Cl^- in the sample solution. Reagent B contains diphenylcarbazone that binds Hg^{2+} to form a purple complex, Hg–diphenylcarbazone, which has an absorption maximum at 550 nm. Therefore, the decrease of absorbance from the blank (no chloride added) is proportional to the chloride concentration in the sample solution.

Reagents

Reagent A: 0.000202 *N* Hg $(NO_3)_2$ in 5 m*M* HNO_3. Mercuric nitrate dihydrate, 1.7132 g, was dissolved in approximately 20 mL water and 0.25 mL concentrated nitric acid and then made to 1 L to produce a 0.01 *N* solution. The exact normality of the mercuric nitrate solution was determined by titration using 0.01 *N* sodium chloride as standard and diphenylcarbazone as an indicator *(5).* Then, the required amount of the nominal 0.01 *N* mercuric nitrate solution was diluted with nitric acid and water to make 0.000202 *N* Hg $(NO_3)_2$ in 5 m*M* HNO_3. The normality of the mercuric nitrate solution must be slightly higher than 0.0002 *N* to exceed the highest chloride standard that will be used in the final reaction mixture (chloride, 100 µ *M*).

Reagent B: 0.4 g/L diphenylcarbazone. Diphenylcarbazone, 0.5 g, was dissolved in 100 mL of 95% ethanol as a stock solution. Then, 8 mL of the stock solution was diluted with water to 100 mL.

Chloride stock standard solution, 0.01 *N.* Sodium chloride, 0.5845 g, was dissolved in water and made up to 1 L. Working standards ranging from 0.5 to 4 m*M* were made from the stock standard solution.

Procedure

1. A 25 µL sample solution ranging from approximately 0.5 to 3.5 m*M* chloride or standard solution (0, 1, 2, 3, and 4 m*M* chlo-

ride) was placed in two 5-mL glass test tubes. One test tube was labeled "Test" and the other "Spike." For the "Blank" test tube, 25 µL of water was added.
2. Water, 25 µL, or 1 m*M* NaCl solution was added to the "Test" or "Spike" tube, respectively. Water, 25 µL, was added to "Blank" tube.
3. Reagent A, 500 µL, was added to the tubes. The tubes were mixed and incubated for 2 min at room temperature. The incubation time could be extended.
4. Reagent B, 500 µL, was added to the tubes. The tubes were mixed with a vortex mixer.
5. Two minutes after the addition of Reagent B, the absorbance at 550 nm was measured.

   Because the solubility in water of the purple color complex of mercuric ion and dipehenylcarbazone is low, the color decreases rapidly because of the aggregation. A 20-s difference in the time interval from 2 min is acceptable to read absorbance. The absorbance for "Blank," "Test," and "Spike" was designated as A_{blank}, A_{test}, and A_{spike}, respectively.
6. The chloride concentration (m*M*) in the sample solution was calculated as

$$(A_{blank} - A_{test}) \; / \; (A_{test} - A_{spike}) \times 1.$$

Reproducibility

To test the reproducibility of the present method, Accutrol Normal (Sigma) (human control serum), human urine, and rat plasma pool as control specimens were analyzed. Within-run reproducibility was tested by the repetitive measurements in a single run. These control specimens were divided into aliquots and the aliquots were stored at –20°C. Immediately after thawing the aliquots, their chloride concentrations were measured four times over several days. The run-to-run reproducibility was based on three "Test" assays and three "Spike" assays in a single run.

Animal Study

Sixteen male Sprague–Dawley rats (Charles River/SASCO, Wilmington, MA) weighing approximately 30 g were equally and randomly divided into two groups and fed normal-salt or high-salt diets containing 2.6 or 82.6 g NaCl/kg diet, respectively. The composition of the normal-salt diet is shown in Table 1. Eighty grams of sucrose in the normal-salt diet was replaced with 80 g sodium chloride for the high-salt diet. After 70 d feeding, urine was collected for 16 h using a metabolic cage. During urine collection, no food was given and rats had free access to purified water (Super Q, Millipore). Urinary chloride excretion was measured by the present method.

Table 1
Composition of Normal-Salt Diet

Ingredient	g/kg diet
Vitamin free casein (Sigma)	**150.00**
Corn starch (Teklad)	**150.00**
Sucrose	**526.50**
Cellulose (Alphacel)	**50.00**
Corn oil	**80.00**
Vitamin mixture	**5.00**
Mineral mixture 1	**17.10**
Mineral mixture 2	**17.90**
L-Cystine	**3.00**
Choline chloride	**0.50**

Note: The vitamin mixture provided 30 mg nicotinic acid, 16 mg calcium panthothenic acid, 6 mg thiamin hydrochloride, 6 mg riboflavin, 7.5 mg pyridoxine hydrochloride, 1 mg folic acid, 0.2 mg D-biotin, 25 µg vitamin B_{12}, 18 mg (18 IU) DL-α-tocopherol acetate, 2.2 mg (4000 IU) retiniyl palmitate, 25 µg (1000 IU) vitamin D_3, and 0.75 mg vitamin K_1 per 1-kg diet.

Mineral mixture 1 provided 12.5 g $CaCO_3$, 2.0 g KCl, and 2.6 g NaCl per 1-kg diet.

Mineral mixture 2 provided 8.8 g KH_2PO_4, 0.8 g MgO, 0.102 g sodium meta-silicate nonahydrate, 125.3 mg ferric sulfate, 10 mg ZnO, 40 mg $MnSO_4{\cdot}4H_2O$, 20 mg $CuSO_4{\cdot}5H_2O$, 0.5 µg KI, 0.6 µg Na_2SeO_3, 2.7 µg ammonium molybdate, 2 mg $Cr(C_2H_3O_2){\cdot}3H_2O$, 6 mg H_3BO_3, 6 mg NaF, 0.5 mg NH_4VO_3, 2 mg $Na_2HAsO_4{\cdot}7H_2O$, and 4.05 mg $NiCl_2{\cdot}6H_2O$ per 1-kg diet.

RESULTS AND DISCUSSION

Figure 1 shows the calibration curve of the chloride colorimetry. The chloride concentration indicated was in the final reaction mixture. The absorbance decreased as the chloride concentration increased, because this method uses reciprocal colorimetry. The calibration curve was linear within the range 0–100 µ*M* (r = –0.9997). An absorbance change of 1.0 typically corresponds to chloride concentration of 100 µ*M*

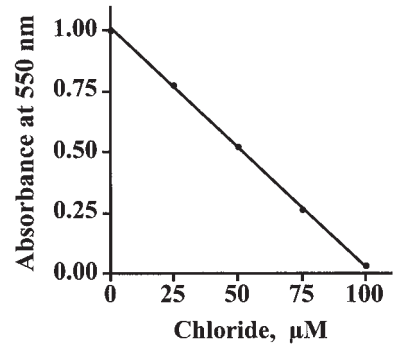

Fig. 1. Calibration curve for chloride colorimetry. The concentration of chloride at the abscissa shows the chloride concentration in the final reaction mixture. The absorbance at the ordinate is proportional to the produced mercury–diphenylcarbazone purple complex. The correlation coefficient is –0.9997 (n = 5). Slope is –0.00982 absorbance/μ*M;* y-intercept-1.011 absorbance.

in the final reaction mixture (4 m*M* in the sample solution). It is possible to increase the concentrations of Reagents A and B, decrease the volume of Reagents A and B, and increase the volume of the sample. This modification will increase the apparent sensitivity of the method based on the chloride concentration in the sample, although the sensitivity does not change based on chloride concentration in the final reaction mixture. Six replicate measurements of the blank (0 μ*M*) solution gave 1.023±0.008 (mean±SD) of the absorbance. The 3σ detection limit of the chloride in the final reaction mixture was 2 μ*M.* A typical detection limit of the sophisticated instrumental analysis of chloride is 0.2 mg/L (5.6 μ*M*) for ion chromatography *(13)* and 4 μg/L (0.11 μ*M*) for capillary electrophoresis *(14).* Fifty micromolars of chloride in the final reaction mixture gave 0.532±0.005 (mean±SD) of the absorbance (n = 6) with 0.9% of the coefficient of variation. The quantitation range was 0.4–4 m*M* in the sample solution when the limit of quantitation was defined to be the concentration that provides 0.1 of absorbance.

Chloride concentrations in urine specimens are sometimes quite low compared to plasma concentrations that can contain about 100 m*M* chloride. For example, in the short-term water load test applied to the same rats (the precise procedure not reported here), 1.1 m*M* was the lowest chloride concentration in rat urine that was diluted at the sample collection by the addition of water to compensate the small urine volume. The sensitivity of this method allowed the chloride assay in these diluted rat urine samples. The chloride concentration in the diluted rat samples was far less than the quantitation range of other established spectrophotometric methods of chloride, typically 80–125 m*M (7)* and 15–180 m*M (15).* The mercuric titration method for routine assay reported by Annino *(6)* uses microburets with 0.01 mL of the smallest increment. The volume of titrant with accept-

Table 2
Within-run and run-to-run reproducibility of the present method

	Within-Run CV	Run-to-Run CV
Human serum (Accutrol™ Normal, Sigma)	4.0 % (n = 15)	4.7 % (n = 4)
Human urine (custom-made pool)	2.3 % (n = 12)	3.7 % (n = 4)
Rat plasma (custom-made pool)	2.6 % (n = 12)	5.3 % (n = 4)

CV: Coefficient of variation.

Human serum (Accutrol™ Normal, Sigma), human urine and heparinized rat plasma were separated into aliquots and stored at –20°C to test the run-to-run reproducibility. Each run used three "Test" assays and three "Spike" assays for run-to-run reproducibility test.

able accuracy (such as 2%) for microburets is 0.5 mL. When 10 times higher volume of urine sample is used with replacing 2 mL water added to an Erlenmeyer flask for titration, the lower limit of measurement becomes approximately 5 m*M*.

Because biological samples (i.e., human and rat plasma and urine) interfered with color development, the standard addition method was employed for the correction of interference. The analyzed value of chloride concentration in the human control serum Accutrol Normal (Sigma) was 101±4 m*M* (mean±SD, n = 12). The certified value of chloride in Accutrol Normal by Sigma is 102 m*M* with a mean in the range 91–113 m*M* based on the colorimetric method of Schoenfeld and Lewellen *(1)*. Table 2 shows the within-run and run-to-run (measured over several days) reproducibilities for human and rat samples.

The urinary excretion rate of chloride was 23.6±9.3 µmol/h (mean±SD, n = 8) and 126.2 ± 28.0 µmol/h (n = 8) for rats fed a normal-salt diet and a high-salt diet, respectively. The urinary excretion of chloride in rats fed the high-salt diet was significantly higher than that for rats fed the normal diet ($p < 0.001$) by Welch's t-test.

The use of mercuric compounds may produce the potential problem of disposal of reagents and wastes. The utilization of mercury can be justified because mercuric compounds are still in use in the mercuric nitrate titration method and some commercial kits for chloride determination *(7)* and there are benefits of this method enabling the measurement of low chloride concentrations in animal urine samples without sophisticated instrumentation.

In conclusion, the present method is sensitive and suitable for determining chloride concentration in biological samples, especially for rat urine which sometimes contains lower concentrations of chloride than can be accurately measured with the colorimetric method based on the iron–thiocyanate complex or the titration method.

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